

Determination of Biologically Relevant Vitamin D Metabolites in a Mouse Model of Non Melanoma Skin Cancer

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INTRODUCTION

Vitamin D: the sunshine vitamin.

Vitamin D is a unique nutrient in that it is not only obtained from food, but it is also produced in appreciable quantities in the skin. Endogenous production of vitamin D begins with UVB irradiation, which forces the B-ring of steroidal pro-vitamin D to break at the ninth carbon. Pre-vitamin D compounds are then isomerized by heat to form vitamin D ¹. The dietary guidelines for vitamin D consumption have recently been revised by the Institute of Medicine and raised to 600-800 International Units (IU)/day, depending on age ².

Whether ingested through the diet, or made in the skin, vitamin D is readily hydroxylated by 25 hydroxylase to 25-hydroxyvitamin D (25OH D) in the liver and deposited into circulation attached to the vitamin D binding protein. Further hydroxylation of 25OH D at the first carbon by CYP27B1 results in the formation of 1,25-dihydroxyvitamin D (1,25(OH)₂ D). This metabolite is considered the active form of vitamin D because it directly binds to the nuclear vitamin D receptor, which regulates numerous transcription factors. The formation of 1,25-dihydroxyvitamin D is tightly regulated, and as such, this metabolite subsists at low levels approximated at 1000 times less than that of 25OH D ³. 25OH D and 1,25-dihydroxyvitamin D

are both rendered inactive by CYP24, which hydroxylates these metabolites at the twenty-fourth carbon.

Vitamin D and Non-Melanoma Skin Cancer

Non melanoma skin cancer (NMSC) is the most prevalent type of cancer in the United States with approximately 2.2 million people diagnosed each year ⁴. Although this disease is not particularly deadly, the cost incurred through treatment of the disease is a severe burden for those affected ⁵. The most easily modifiable risk factor for NMSC is unprotected UV exposure, yet some researchers suggest that individuals receive approximately 5-15 minutes of unprotected sun exposure three times a week for sufficient vitamin D production ⁶. Other researchers suggest that moderate unprotected sun exposure allows for sufficient production of vitamin D without significantly increasing a person's risk for skin cancer ⁶. Still, it is reasonable to expect that individuals following such advice will remain in the sun for longer than necessary, increasing the risk of developing UV-induced skin cancer ⁷.

The role of vitamin D in NMSC is also not well known. Most of the studies investigating the relationship between vitamin D and NMSC focus on basal cell carcinoma (BCC), one of the subtypes of NMSC. Two prospective studies investigating the relationship of several dietary nutrients and BCC concluded that dietary vitamin D had no protective effect against BCC ^{8,9}. It should be noted that these studies did not measure serum 25OH D levels, and thus lack a proper biomarker to verify vitamin D intake and status. Asgari and colleagues ¹⁰ reported an increased risk for BCC with higher prediagnostic serum 25OHD levels in a nested case control study. Epidemiological studies investigating the relationship between vitamin D status and general NMSC, including BCC and squamous cell carcinoma (SCC), have found conflicting results.

Tang and colleagues ¹¹ found that higher baseline 25OH D serum levels coincided with a decreased risk for self-reported NMSC, while Eide et al ¹² reported an increased risk of histologically confirmed NMSC with higher baseline 25OH D levels. Epidemiological studies on this subject are challenging in that it is difficult to control for sun exposure as a confounding factor since it contributes to both vitamin D status and NMSC risk.

Cell and animal studies, while not conclusive, have provided some intriguing evidence on the role of vitamin D in NMSC. Much of the interest in this interaction stems from the fact that keratinocytes, melanocytes, and even cancer cells contain cellular machinery related to vitamin D, including the vitamin D receptor and metabolizing enzymes ^{13,14}. Traditionally it has been assumed that 1,25(OH)₂ D has pro-differentiating and anti-proliferating activities in healthy keratinocytes, while SCC cells lose sensitivity to the pro-differentiating signaling of 1,25(OH)₂ D. However, it has been suggested that the concentration of 1,25(OH)₂ D may also influence cell growth, with lower concentrations exhibiting a pro-proliferative effect in healthy skin cells and high doses having the opposite effect ¹⁰. There is even conflicting evidence on whether or not topical application of 1,25(OH)₂ D is anti- or pro-proliferative ^{15,16}. There is a decent amount of evidence on the ability of 1,25(OH)₂ D to protect skin cells against many types of UV-induced DNA damage ¹⁷. The modulation of the DNA damage response by 1,25(OH)₂D has been recently reviewed by Bikle ¹⁸. In all, the evidence for a role of vitamin D in NMSC is still rather inconclusive.

Most investigators focus on the utility of pure 1,25(OH)₂ D against NMSC rather than dietary vitamin D. This is not surprising, as epidemiological evidence has suggested that the effects of dietary vitamin D are insignificant, especially when compared to the experimentally

verified effects of $1,25(\text{OH})_2 \text{D}$ ¹⁴. However, no animal or human trial has yet investigated the effects of dietary vitamin D on the development of NMSC.

Vitamin D Analysis

The most commonly measured metabolites of vitamin D are the 25-hydroxylated forms of vitamins D_2 and D_3 . The additive concentration of these two metabolites yields the clinically relevant measurement of vitamin D status. Although it is not the active form of vitamin D, total serum 25OH D level is considered the best measurement of status for several reasons. Vitamin D produced endogenously or absorbed from the diet is rapidly converted to 25OH D , and the half-life of this metabolite is approximately 15 days, compared to 15 hours for the active $1,25(\text{OH})_2 \text{D}$ metabolite ¹⁹. Additionally, 25OH D circulates at much higher concentrations than $1,25(\text{OH})_2 \text{D}$ and may be less influenced by other factors ³.

Determination of vitamin D status has been a notable challenge in clinical laboratories, as there is great discrepancy not only between methods used for measurement, but also between laboratories performing the assays. The common methodology used to measure vitamin D status, as well as issues with the current assays, have recently been reviewed by Su and colleagues ³. The four most common methods used for 25OH D quantification include vitamin D binding protein-based competitive protein-binding assays, immunoassays, and high performance liquid chromatography (HPLC) assays with UV or mass spectral detection. Competitive binding protein assays and immunoassays are popular mainly due to their ease and affordability. However, these techniques are susceptible to overestimation of 25OH D_3 due to interfering metabolites of vitamin D_3 , especially C3-epi 25OH D_3 (C3epi), and underestimation of 25OH D_2 due to lower binding affinity ^{3,20,21}. Techniques involving HPLC may separate some

interfering compounds from 25OH D. Thus, among all these methods, it is generally agreed that HPLC coupled with tandem mass spectrometry is the preferred method for vitamin D analysis ²².

In order to further increase the sensitivity of HPLC-MS/MS methods, several groups have begun employing chemical derivatization with Cookson-type reagents. These reagents are 4-substituted 1,2,4-triazoline-3,5-dienes that react with conjugated diene systems in a Diels-Alder fashion ²³. Derivatizing vitamin D metabolites with these agents increase ionization efficiency and provide more specific and predictable mass transitions ^{23,24}. A number of Cookson-type reagents have been suggested for use in vitamin D analysis ²⁰, but the most prevalent, and possibly the most commercially available, is 4-phenyl-3H-1,2,4-triazole-3,5(4H)-dione (PTAD). PTAD has been used successfully to profile vitamin D metabolites in serum ²⁵⁻²⁷, and soft tissues ²⁸, but other mediums, such as murine skin, have been left unexplored. Additionally, no current vitamin D analysis method utilizing PTAD derivatization has reported the separation of 25OH D₃ and C3epi.

The objectives of this study were to develop methodology appropriate for the measurement of vitamin D metabolites in murine skin, and apply the developed method to quantify 25OH D₃ levels in the skin of mice from a study investigating the effects of dietary vitamin D₃ on NMSC.

MATERIALS AND METHODS

Samples

To study the effects of dietary vitamin D on the occurrence of NMSC, the Skh-1 mouse model was used. This model is well-accepted model for the study of NMSC, and more specifically SCC ²⁹. Male (n=75) and female (n=75) mice were fed 25, 150, or 1000 IU vitamin

D₃ in their chow (n=25/sex/diet). After four weeks, these groups were further divided into treatment groups with n=15/sex/diet receiving one minimal erythemal dose of UVB irradiation three times per week and n=10/sex/diet receiving no UV treatment for 25 weeks. Dorsal skin from n=3 mice from each sex/diet/treatment group (total n=36) was analyzed for vitamin D metabolites.

Sample Preparation

Skin samples (0.30 g) were frozen with liquid nitrogen, crushed to a fine powder, and transferred into pre-weighed glass vials. Samples were then spiked with 20 µL of a d₃25OH D₃ solution (100 nmol/L) and extracted with the solvent system used by Stahl and others³⁰ with slight modifications³¹. Briefly, 1 mL of ethanol containing 0.1% butylated hydroxytoluene, 1 mL of water, and 5 mL of 5:1 hexane/dichloromethane were added to the skin samples, and the mixture was probe soicated for 30 s. The homogenized solutions were centrifuged at 2000 x g for 5 min to hasten phase separation, and the upper organic layer was decanted into clean glass vials. The extraction was repeated another two times, with the addition of 5 mL 5:1 hexane/dicholormethane, and the pooled organic layers were dried under a stream of nitrogen. Standards of 25OH D₃ ranging in concentration from 7.80 to 250 nmol/L were spiked with 20 µL of the internal standard solution, dried, and processed alongside extracted skin samples as described below.

Dried extracts were derivatized using 4-Phenyl-1,2,4-triazole-3,5-dione (PTAD) according to Lipkie et al²⁸. 100 µL of a 2 mg/mL PTAD solution were added to the extracts, which were then mixed on a vortex for 10 min. An additional 100 µL of PTAD was added to the solution and mixing was repeated for another 10 min. The reaction was quenched by adding 20

μL of water and mixing for 5 additional minutes. The reaction mixture was dried under nitrogen, reconstituted with 100 μL of acetonitrile, and centrifuged at 15,000 x g for 2 min prior to analysis.

HPLC-MS/MS Analysis

Vitamin D metabolites were separated by HPLC using a Luna C18 reversed phase column (4.6x250 mm, 5 μm; Phenomenex, Torrance, CA) maintained at 40 °C. The binary mobile phase consisted of water (A) and acetonitrile (B), both containing 0.1% formic acid. Optimal separation of vitamin D metabolites was achieved using a step gradient of 29% A for 3.5 min and an immediate switch to 20% A for 3 min, followed by a column wash with 100% B for two minutes and reconditioning at initial conditions for 3.5 min. The injection volume was 10 μL and the injection needle was washed with acetonitrile between runs. Eluent from the HPLC was directed to a QTRAP 5500 mass spectrometer (AB Sciex; Framingham, MA) equipped with atmospheric pressure chemical ionization (APCI) operated in positive ion mode. MS parameters were as follows: source temperature, 450 °C; curtain gas, 30 psi; ion source gas 1, 60 psi; ion source gas 2, 0 psi; declustering potential, 185 V; entrance potential, 10 V; collision cell exit potential, 11 V. Specific MS parameters are elaborated in table 1.

25OH D₃ and C3-epi-25OH D₃ were quantified using isotope dilution methodology with Analyst 1.5.1 (ABSciex; Foster City, CA). Briefly, standards with varying concentrations of 25OH D₃ were spiked with a constant concentration of d₃25OH D₃ and processed as described above. The areas under the peaks representing derivatized 25OH D₃ and its labeled counterpart were calculated, and a standard curve was constructed using the ratio of labeled to unlabeled

standard. 25OH D₃ and C3-epi-25OH D₃ in the skin extracts were quantified using this standard curve.

Table 1. Relevant parameters used for the analysis of vitamin D metabolites

Analyte	Retention Time (min)	Mass Transition	Collision Energy (V)	Dwell Time (ms)
PTAD-25OH D ₃	5.9	558.3 > 298.3	25	280
PTAD-C3-epi-25OH D ₃	6.2	558.3 > 298.3	25	280
PTAD-d ₃ 25OH D ₃	6.3	561.3 > 301.3	25	280

Statistical Analysis

Statistical significance (p<0.05) was calculated using multivariate analysis and Tukey's post-hoc test in SPSS (IBM, Armonk, NY).

RESULTS AND DISCUSSION

The first objective of this work was to develop methodology appropriate for the analysis of vitamin D metabolites in skin. The metabolites successfully profiled in skin were 25OH D₃ as well as the C3-epimer of this compound (C3epi). To our knowledge, this is the first time that this analysis has been performed in murine skin, and we are the first to chromatographically separate PTAD-derived 25OH D₃ and C3-epi for quantification. The separation of these PTAD-derived metabolites was challenging due to the fact that derivatization with PTAD results in the formation of (R) and (S) enantiomers of each derived compound³². The example chromatogram of PTAD-25OH D₃ and PTAD-C3epi standards (figure 1) details the elution pattern of these compounds on the Luna C18 column. The two enantiomers of PTAD-C3epi co-elute between

the two enantiomers of PTAD-25OH D₃. The method was found to be sufficiently sensitive for the detection and quantification of these metabolites in murine skin.

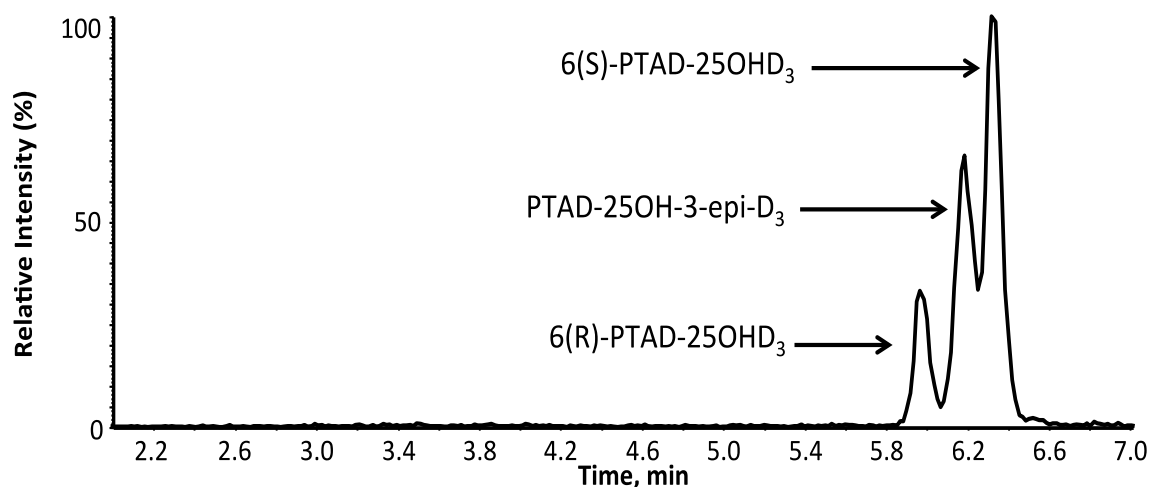


Figure 1. Example chromatogram of PTAD-derivatives of 25OH D₃ and C3epi standards

The developed method was applied to measure vitamin D metabolites in a study of effects of dietary vitamin D on NMSC in mice. In this study, mice were fed increasing amounts of vitamin D while being exposed or remaining unexposed to UVB irradiation. Since vitamin D is produced via UVB irradiation of the skin, vitamin D status (25OH D₃) in the skin was expected to be greater for those mice receiving the UVB treatment than those not receiving the treatment. Surprisingly, no significant differences from this treatment were noticed in these mice. However, a dose-dependent response was detected regardless of UV exposure. As shown in figure 2, the 25OH D₃ level in the skin reflected increases in dietary vitamin D₃ with statistical significance between each dose. Interestingly, C3epi levels increased significantly only at the highest dose, suggesting that this metabolite may become more important in a high-vitamin D state.

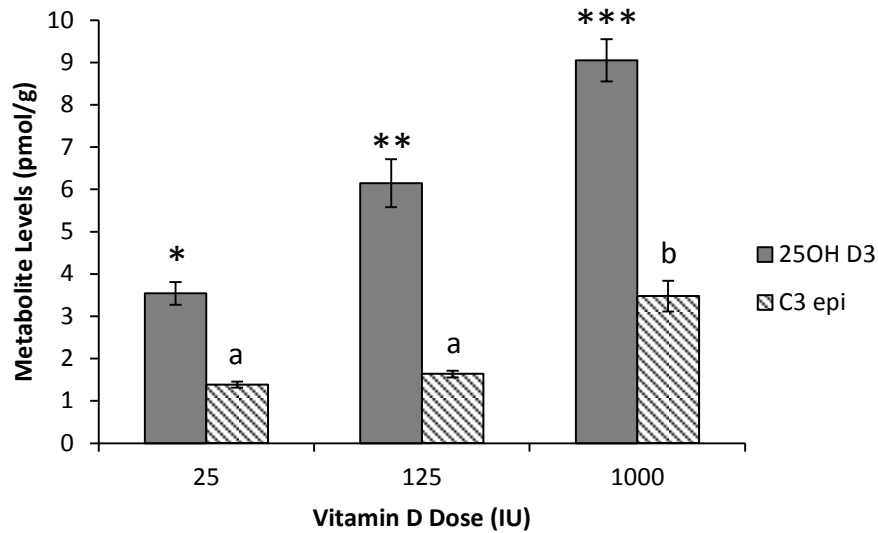


Figure 2. Vitamin D metabolite levels in skin of mice fed increasing doses of vitamin D₃. Significant differences are denoted by * and lower case letters for 25OH D₃ and C3epi, respectively. Error bars represent standard error. (n=12 mice/dose).

Apart from differences between the skins of mice fed increasing amounts of vitamin D₃, few other significant differences appeared in this sample set. However, when examining the dose-response data with regard to sex, there was a significant difference in the C3epi levels of male and female mice fed 1000 IU vitamin D₃. No other differences were noticed between male and female mice in this work. The metabolite levels of male and female mice at each dose level are shown in figure 3. Though the difference between males and females was slight, it is still interesting, as sex is believed to influence NMSC. Females tend to develop less NMSC than males⁷. Remarkably, the levels of 25OH D₃ were similar for both males and females at all three doses of vitamin D₃.

The data collected in this work will be used to help explain the results seen in the histology and other cancer outcomes of this mouse study. Future work will include serum measurements of 25OH D₃ and C3epi from the same mice. The method that was developed for this analysis is expected to have a strong impact on future work with vitamin D. As researchers

become more interested in low-abundant metabolites of vitamin D in the tumor micro-environment, sensitive methods that are able to separate derived 25OH D₃ and C3epi will be vital for accurate measurements of these compounds.

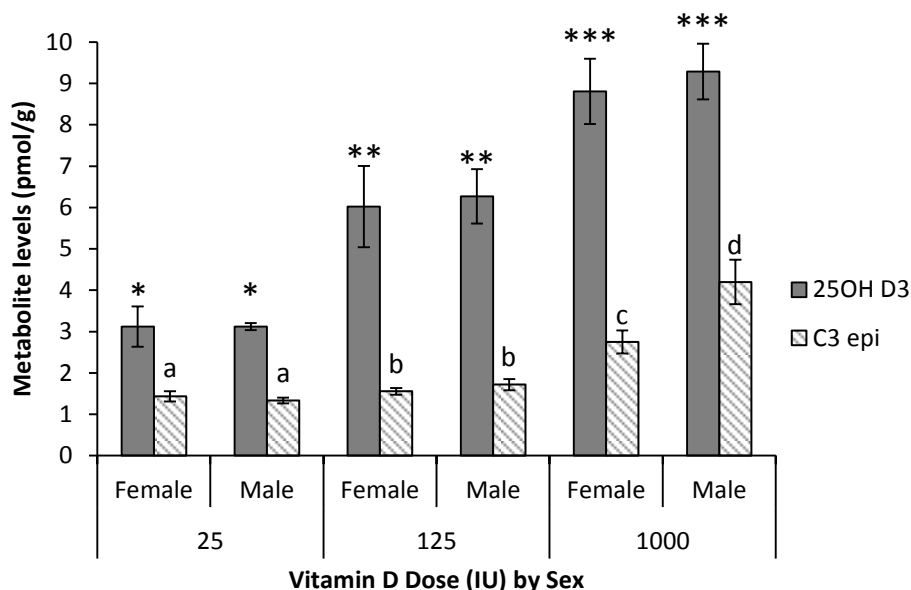


Figure 3. Vitamin D metabolite levels in skin of male and female mice fed increasing doses of vitamin D₃. Significant differences are denoted by * and lower case letters for 25OH D₃ and C3epi, respectively. Error bars represent standard error. (n=6 mice/dose/sex).

Conclusions

We have shown, for the first time, a dose-dependent increase of 25OH D₃ levels in skin from mice fed increasing amounts of vitamin D₃. Additionally, differences in C3epi levels of male and female mice fed 1000 IU vitamin D₃ were found. These measurements were possible due to a newly developed method which chromatographically separates PTAD-derivatized 25OH D₃ and C3epi. The results of this study will no doubt inform future studies of the effects of dietary vitamin D on NMSC and facilitate more accurate measurements of derivatized 25OH D₃ levels.

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References

- (1) Holick, M. F. In *Vitamin D: Physiology, Molecular Biology, and Clinical Applications*; Holick, M. F., Ed.; Springer Science+Business Media: New York, NY, 2010; pp. 3–34.
- (2) Institute of Medicine of the National Academies. *Dietary Reference Intakes for Calcium and Vitamin D*; Ross, A. C.; Taylor, C. L.; Yaktine, A. L.; Del, H. B., Eds.; The National Academies Press: Washington, D.C., 2011; p. 1132.
- (3) Su, Z.; Narla, S. N.; Zhu, Y. *Clin. Chim. Acta* **2014**, *433*, 200–205.
- (4) The American Cancer Society. www.cancer.org (accessed Apr 27, 2014).
- (5) Chen, J.; Fleisher, A.; Smith, E.; Kancler, C.; Goldman, N.; Williford, P.; Feldman, S. *Dermatologic Surg.* **2001**, *27*, 1035–1038.
- (6) Holick, M. F. In *Sunlight, Vitamin D and Skin Cancer*; Reichrath, J., Ed.; Springer Science+Business Media: Austin, TX, 2008; pp. 1–15.
- (7) Oberyszyn, T. M. *Cancer Lett.* **2008**, *261*, 127–136.
- (8) Hunter, D. J.; Colditz, G. a; Stampfer, M. J.; Rosner, B.; Willett, W. C.; Speizer, F. E. *Ann. Epidemiol.* **1992**, *2*, 231–239.
- (9) Van Dam, R. M.; Huang, Z.; Giovannucci, E.; Rimm, E. B.; Hunter, D. J.; Colditz, G. a; Stampfer, M. J.; Willett, W. C. *Am. J. Clin. Nutr.* **2000**, *71*, 135–141.
- (10) Asgari, M. M.; Tang, J.; Warton, M. E.; Chren, M.-M.; Quesenberry, C. P.; Bikle, D.; Horst, R. L.; Orentreich, N.; Vogelman, J. H.; Friedman, G. D. *J. Invest. Dermatol.* **2010**, *130*, 1438–1443.
- (11) Tang, J. Y.; Parimi, N.; Wu, A.; Boscardin, W. J.; Shikany, J. M.; Chren, M.-M.; Cummings, S. R.; Epstein, E. H.; Bauer, D. C. *Cancer Causes Control* **2010**, *21*, 387–391.
- (12) Eide, M. J.; Johnson, D. a; Jacobsen, G. R.; Krajenta, R. J.; Rao, D. S.; Lim, H. W.; Johnson, C. C. *Arch. Dermatol.* **2011**, *147*, 1379–1384.

- (13) Mason, R. S.; Reichrath, J. *Anticancer. Agents Med. Chem.* **2013**, *13*, 83–97.
- (14) Bikle, D. D.; Oda, Y.; Xie, Z. *J. Steroid Biochem. Mol. Biol.* **2005**, *97*, 83–91.
- (15) Lutzow-Holm, C.; De Angelis, P.; Grosvik, H.; Clausen, O. *Exp. Dermatol.* **1993**, *2*, 113–121.
- (16) Dixon, K. M.; Norman, A. W.; Sequeira, V. B.; Mohan, R.; Rybchyn, M. S.; Reeve, V. E.; Halliday, G. M.; Mason, R. S. *Cancer Prev. Res. (Phila)*. **2011**, *4*, 1485–1494.
- (17) Gordon-Thomson, C.; Gupta, R.; Tongkao-on, W.; Ryan, A.; Halliday, G. M.; Mason, R. S. *Photochem. Photobiol. Sci.* **2012**, *11*, 1837–1847.
- (18) Bikle, D. D. *Photochem. Photobiol. Sci.* **2012**, *11*, 1808–1816.
- (19) Jones, G. *Am. J. Clin. Nutr.* **2008**, *88*, 582S–586S.
- (20) El-Khoury, J. M.; Reineks, E. Z.; Wang, S. *Clin. Biochem.* **2011**, *44*, 66–76.
- (21) Wallace, A. M.; Gibson, S.; de la Hunty, A.; Lamberg-Allardt, C.; Ashwell, M. *Steroids* **2010**, *75*, 477–488.
- (22) Zerwekh, J. E. *Am. J. Clin. Nutr.* **2008**, *87*, 1087S–1091S.
- (23) Shimada, K.; Oe, T.; Mizuguchi, T. *Analyst* **1991**, *116*, 1393–1397.
- (24) Higashi, T. H.; Miura, K. M.; Kitahori, J. K.; Shimada, K. S. *Anal. Sci.* **1999**, *15*, 619–623.
- (25) Aronov, P. a; Hall, L. M.; Dettmer, K.; Stephensen, C. B.; Hammock, B. D. *Anal. Bioanal. Chem.* **2008**, *391*, 1917–1930.
- (26) Duan, X.; Weinstock-Guttman, B.; Wang, H.; Bang, E.; Li, J.; Ramanathan, M.; Qu, J. *Anal. Chem.* **2010**, *82*, 2488–2497.
- (27) Ding, S.; Schoenmakers, I.; Jones, K.; Koulman, A.; Prentice, A.; Volmer, D. a. *Anal. Bioanal. Chem.* **2010**, *398*, 779–789.
- (28) Lipkie, T. E.; Janasch, A.; Cooper, B. R.; Hohman, E. E.; Weaver, C. M.; Ferruzzi, M. G. *J. Chromatogr. B* **2013**, *932*, 6–11.
- (29) Benavides, F.; Oberyszyn, T. M.; VanBuskirk, A. M.; Reeve, V. E.; Kusewitt, D. F. *J. Dermatol. Sci.* **2009**, *53*, 10–18.
- (30) Stahl, W.; Schwarz, W.; Sundquist, a R.; Sies, H. *Arch. Biochem. Biophys.* **1992**, *294*, 173–177.

- (31) Kopec, R. E. Bioavailability, Metabolism, and Bioefficacy of Tomato Carotenoids, The Ohio State University, 2012, p. 184.
- (32) Higashi, T.; Shibayama, Y.; Fuji, M.; Shimada, K. *Anal. Bioanal. Chem.* **2008**, *391*, 229–238.